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PHOSPHORYLATED EXTRACELLULAR SIGNAL-REGULATED KINASE 1/2 IMMUNOREACTIVITY IDENTIFIES A NOVEL SUBPOPULATION OF SYMPATHETIC PREGANGLIONIC NEURONS

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Abstract—Distinct chemical codes are thought to reflect functional specificity in sympathetic preganglionic neurons (SPN). Although a number of chemical candidates have been identified including neurotransmitter-related, calcium-binding and other proteins, signal transduction proteins have been largely neglected. Not only might these chemicals allow discrimination of functionally unique chemical signatures, but they may also identify activated neurons.

Immunoreactivity (ir) to phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) was differentially located within the thoracic spinal cord depending upon which of three forms of killing was used: the only exception to this was the intermediolateral cell column (IML) which was consistently, densely labeled. The presence or absence of p-ERK1/2 in SPN (n=17,541) within the IML of the thoraco-lumbar spinal cord was determined in seven rats. SPN were identified on the basis of their location, size and that they contained choline acetyltransferase ir. On average, 58% of SPN contained p-ERK1/2, however, more SPN in both the upper (72%; C8–T4) and lower (78%; T11–L3) thoraco-lumbar spinal cord contained p-ERK1/2-ir than the middle thoracic region (47%; T4–T10). p-ERK1/2-ir was also examined in SPN (n=1895) innervating the adrenal medulla (identified by retrograde tracing using cholera toxin B subunit) combined with localization of neuronal nitric oxide synthase (nNOS) in three rats. On average, 64% of adrenal SPN contain p-ERK1/2-ir, and it was confirmed that all adrenal SPN contain nNOS-ir. It appears that p-ERK1/2-ir SPN, described in this study, have tonically activated receptors that are coupled to intracellular signal transduction pathways that lead to the phosphorylation of ERK1/2. **© 2005 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: signal transduction proteins, adrenal medulla, nitric oxide, tonic activity.

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Abbreviations: ChAT, choline acetyltransferase; CTB, cholera toxin B subunit; ERK1/2, extracellular signal-regulated kinase 1/2; IML, intermediolateral cell column pars principalis; ir, immunoreactivity; NHS, normal horse serum; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; PB, phosphate buffer; p-ERK1/2, phosphorylated extracellular signal-regulated kinases 1 and 2; SPN, sympathetic preganglionic neurons; TPBS, Tris–phosphate-buffered saline.

Sympathetic preganglionic neurons (SPN) are the final central neurons that control the sympathetic nervous system. In the rat, they are situated in the thoraco-lumbar spinal cord, with approximately 85% located in the intermediolateral cell column (IML) and the rest distributed in the central autonomic and intercalated nuclei and the dorsolateral funiculus (Strack et al., 1988). There is now considerable evidence that SPN are organized into functionally discrete pathways in the spinal cord and are differentially controlled according to the targets they regulate (Strack et al., 1988). It is proposed that these specific populations of SPN are "chemically coded" (Gibbins, 1992; Janig and McLachlan, 1992) such that SPN of a particular functional class may be discriminated from other SPN according to unique profiles of neurochemicals that they express (Costa et al., 1986; Grkovic et al., 1999; Chanthaphavong et al., 2003).

Identifying such chemical coding is difficult as each segment of the spinal cord contains functionally heterogeneous populations of SPN (Pilowsky et al., 1992; Boczek-Funcke et al., 1992; Jansen et al., 1993). SPN innervating the adrenal gland, however, are readily identified as they have a direct neuroanatomical projection to the target organ, rather than to functionally heterogeneous ganglia. Moreover, it appears that the populations of SPN innervating the adrenal gland may have discrete chemical codes. While all adrenal SPN contain acetylcholine (ACh) and neuronal nitric oxide synthase (nNOS) (Blottner and Baumgarten, 1992; Vogel et al., 1997; Murphy et al., 2003), only SPN innervating noradrenergic chromaffin and adrenal ganglion cells contain calretinin (Afework and Burnstock, 1995), and only SPN innervating adrenergic chromaffin cells contain enkephalin (Holgert et al., 1995). The chemical coding of most functionally defined sympathetic pathways remains to be defined, strongly indicating a need to identify alternate makers to discriminate different populations of SPN. Another group of cellular proteins that may be useful in differentiating subpopulations of neurons, but is as yet unexplored, includes those involved in signal transduction. Signaling proteins such as extracellular signal-regulated kinases 1 and 2 (ERK1/2) or cyclic AMP response element binding protein (CREB) may be useful not only as chemical markers, but also when identified in their phosphorylated forms (p-ERK1/2, pCREB) as activity-dependent markers. This approach is now being used widely in the nervous system (Ginty et al., 1993; Chan et al., 1999; Reijmers et al., 2000) and has a variety of advantages: phosphorylation occurs within minutes compared with the emergence of activity driven proteins such

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as Fos which occurs within hours (Xia et al., 1996); both inhibitory and excitatory inputs can phosphorylate the protein (Ortiz et al., 1995; Vanhoose et al., 2002); and signal transduction pathways that are activated in response to functionally specific stimuli can be determined (Lindgren et al., 2002; Kawasaki et al., 2004). Phosphorylated proteins may be particularly useful in distinguishing functionally specific SPN populations with ongoing activity, such as those involved in cardiovascular regulation, from those that are silent unless activated by particular stimuli, such as those supplying sweat glands or hairy skin (Janig and McLachlan, 1992).

Although the identification of phosphorylated proteins is a rapidly expanding field the effects of different forms of killing have received little attention (but see Gioia et al., 2003). General anesthetics mediate their effects by acting at neurotransmitter receptors within the CNS (Antkowiak, 2001) and thus may excite, inhibit, disfacilitate or disinhibit neurons potentially phosphorylating or dephosphorylating proteins.

The present study had three main aims. First, to assess the effects of three forms of killing (halothane, sodium pentobarbital, cervical dislocation) on the distribution of p-ERK1/2 immunoreactivity (ir) in the thoracic spinal cord. Second, to determine which, if any, SPN in the IML of the thoraco-lumbar spinal cord contain p-ERK1/2-ir. This was achieved using choline acetyltransferase (ChAT) ir as a marker for SPN combined with an antibody directed against p-ERK1/2. Our third aim was to examine the distribution of p-ERK1/2-ir in a functionally defined population of SPN viz. those supplying the adrenal gland. The retrograde tracer cholera toxin B subunit (CTB) was used to reveal the population of SPN innervating the adrenal gland and the presence or absence of nNOS-ir and p-ERK1/2-ir was determined in this population.

EXPERIMENTAL PROCEDURES

Animals

Twenty adult male Sprague–Dawley rats (300–400 g) were used in this study (Gore Hill Laboratories, RNSH, Sydney, Australia). All protocols were approved by the Animal Care and Ethics Committee of the Royal North Shore Hospital and conducted in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes (http://www.health.gov.au:80/nhmrc/research/awc/code.htm). Every effort was made to minimize pain or discomfort experienced by the animals.

Ten animals were used to evaluate the distribution of p-ERK1/2-ir in the thoracic spinal cord following three forms of killing. A further 10 animals were used to quantitatively evaluate the distribution of p-ERK1/2-ir in SPN in the IML of the thoracic spinal cord and determine the presence of chemical coding in a functionally defined subpopulation of SPN.

Killing

In three animals anesthesia was initially induced, via a mask, with 4% halothane in oxygen and was reduced to 3% following the loss of the righting reflex. Four animals were administered an overdose of sodium pentobarbital (100 mg/kg, i.p.; Nembutal®, Merial, Sydney, NSW, Australia). The time from onset of anesthesia to perfusion was approximately 5 min in both groups. Perfusion was initiated as soon the withdrawal reflex was lost. Three animals were killed by cervical dislocation and perfused immediately (within 2 min). All animals were perfused as described below.

Retrograde tracer (CTB) injections

Adrenal SPN were retrogradely labeled with CTB (1%; C9903; Biological Laboratories, Campbell, CA, USA) in three rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Using a retroperitoneal approach, bilateral applications of CTB (2.5 μl) were pressure injected into the adrenal glands using a glass micropipette with a tip diameter of 100 μm. The wounds were then closed in layers, the rats recovered and housed separately. Three days after surgery CTB-injected rats ($n=3$) as well as untreated rats ($n=7$) were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.). The time-period from induction of anesthesia to loss of withdrawal reflexes was approximately 5 min.

Tissue processing

All 20 animals were perfused in the following manner. Following rapid exposure of the heart a 1 ml solution containing heparin sodium (4500 IU) and sodium nitrite (0.5%) in water was injected into the left ventricle. Rats were then perfused transcardially with 200–350 ml of ice-cold oxygenated tissue culture medium (pH 7.4, DMEM/F12, Sigma D-8900) for about 3 min, followed by 700–800 ml of ice-cold 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4; PB). A peristaltic perfusion pump set to provide constant flow was used. The thoraco-lumbar spinal cord (C8–T6 or C8–L3) was removed and cut into blocks two to five segments long. The tissue was post-fixed in the same fixative overnight at 4 °C and then washed briefly with cold PB (3×30 min). The pieces of spinal cord were cut either parasagittally or coronally. Serial 50 μm sections were cut using a vibrating microtome (Leica, 1000) into PB.

Immunohistochemistry

All sections were incubated in 50% ethanol in deionized water for 30 min immediately after cutting in order to improve antibody penetration. Then, depending on the combinations of antibody applied (Table 1), different processing was undertaken to optimize staining. Sections incubated with antibodies to ChAT and p-ERK1/2 or p-ERK1/2 alone were washed 3×30 min in Tris–phosphate-buffered saline (TPBS; Tris HCL 10 mM, sodium PB 10 mM, 0.9% NaCl, pH 7.4). Sections incubated with antibodies to nNOS, CTB and p-ERK1/2, were washed 2×15 min with Triton X-100 in TPBS (3%; TPBS-X), followed by a 1 h incubation with 10% normal horse serum (NHS). TPBS was used for all subsequent washes between incubation steps for 3×30 min and TPBS with 0.05% Merthiolate (Thimerosal; TPBSm) was used as a diluent for all incubations. During incubation periods, sections were continuously agitated at room temperature. All dilutions of primary antisera were determined by titration in preliminary experiments to give optimal staining with minimal non-specific background. Omission of primary or secondary antibodies resulted in a complete lack of staining.

Coronal sections from C8–T6 were incubated in TPBS containing the p-ERK1/2 antibody (Table 1; study 1) and 5% NHS (CSL Bioscience, Parkville, VIC, Australia) for 72 h at 25 °C. After washing, sections were incubated for 24 h in secondary antibody (Table 1; study 1) with 2% NHS and then, following the washing of sections, incubated overnight in ExtrAvidin–horseradish peroxidase (1:1000, Sigma-Aldrich). Visualization of the reaction product was achieved by incubation with 3,3′-diaminobenzidine tetrahydrochloride, nickel–ammonium sulfate, glucose and glucose oxidase (Pilowsky et al., 1992). Sections were then mounted from 0.5% gelatin on to gelatinised slides, air-dried, dehydrated and delipidated in graded alcohols, chloroform and xylene before being coverslipped (Ultramount).

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